STUDIES ON THE MECHANISM OF PHOSPHATE ACCUMULATION BY SEA URCHIN EMBRYOS [1]

J. B. LITCHFIELD [2] AND A. H. WHITELEY

Department of Zoology and the Friday Harbor Laboratories, University of Washington, Seattle 5, Washington

It was shown by Needham and Needham (1930) that the developing larva of *Dendraster excentricus* increases its total phosphate content from fertilization to gastrulation. Since this initial discovery, phosphate accumulation by echinoderm eggs has been the object of a number of studies. The use of radioactive phosphorus in this analysis was introduced by Brooks (1943).

P^{32} uptake by unfertilized eggs is negligible. An exchange that does not result in an increase in the internal concentration of phosphate is thought to take place between the external and intracellular phosphate (Chambers and White, 1949, 1954; Brooks and Chambers, 1954). Lindberg (1949, 1950) considered that the surface of unfertilized eggs metabolized phosphate but that no intracellular penetration occurred. This surface metabolism involved incorporation of P^{32} into ATP.

The fertilized egg presents a radically different picture. Immediately following fertilization there is no change in P^{32} uptake, but within 6–30 minutes there is a noticeable increase. The rate of uptake increases to a maximum value and remains constant for as much as seven hours after fertilization. These initial events have been described for various species by Abelson (1947), Brooks and Chambers (1948, 1954), Whiteley (1949), and Chambers and White (1954). The maximum uptake rate by the fertilized eggs is as much as 160 times as great as the uptake by unfertilized eggs (Brooks and Chambers, 1948). The rate is not greatly affected by the accompanying decrease in P^{31} and P^{32} concentrations in the suspension medium within the limits 0.7 to 13 μM (Brooks and Chambers, 1954). Evidence that this uptake represents a real penetration is the fact that only 2 to 5% of the P^{32} activity is removed by continuous washing with sea water (Brooks and Chambers, 1948, 1954).

Radioactive phosphate is largely incorporated into the acid-soluble phosphate compounds (Abelson, 1947, 1948; Chambers, Whiteley, Chambers and Brooks, 1948; Chambers and White, 1949, 1954; Bolst and Whiteley, 1957). This is true both in the fertilized and unfertilized eggs. Among the acid-soluble components, the easily hydrolyzable phosphate compounds have the highest activity (Abelson, 1948; Lindberg, 1949, 1950; Chambers and White, 1949, 1954; Bolst and Whiteley, 1957).

Inhibition of uptake by fertilized eggs can be achieved by the use of low tem-

[1] This investigation was supported in part by the Research Fund 171 of the University of Washington, by an Institutional Research Grant from the American Cancer Society to the University of Washington, and by a grant from the National Science Foundation.

[2] Present address: Department of Physiological Chemistry, Woman's Medical College of Pennsylvania, Philadelphia.

peratures (Abelson, 1947; Villee and Villee, 1952). Metabolic inhibitors such as 4,5-dinitro-o-cresol (Abelson, 1947) and cyanide (Brooks and Chambers, 1948) also diminish uptake. These experiments have lead to the view that the developing embryo has an enzymatic mechanism that controls the penetration and consequent accumulation of phosphate. Lindberg (1949, 1950) has adduced evidence that in the fertilized egg the initial enzymatic reaction in the uptake involves incorporation of phosphate into adenosinetriphosphate. However, Chambers and Mende (1953) have determined that the primary penetration of phosphate through the plasma membrane still could be a matter of simple diffusion down an activity gradient inasmuch as they have found an extremely low concentration of free inorganic orthophosphate within the fertilized eggs of *Strongylocentrotus droebachiensis*. Chambers and White (1949) have found that the inorganic phosphate pool in *S. purpuratus* decreases quickly in response to fertilization. Agents that affect enzymatic activity might alter phosphate penetration by affecting the magnitude of the internal phosphate pool.

In the present study the nature of penetration of phosphate into the fertilized egg of *Strongylocentrotus purpuratus* has been examined further. The experiments have largely involved an analysis of the effects of metabolic effectors (2,4-dinitrophenol, arsenate, ATP, and temperature) on the rate of uptake of P^{32} in fertilized eggs.

Methods and Materials

Experimental chamber. The basic experimental approach used in most of the experiments to be described was to measure continuously with a Geiger counter the accumulation of P^{32} by sea urchin eggs that were being perfused with sea water containing P^{32} as orthophosphate. In some experiments the perfusion fluid included various metabolic effectors. The lucite perfusion chamber was an improved version of that used by Chambers and Whiteley (Whiteley, 1949), and Chambers, White and Zeuthen (Zeuthen, 1951) (Fig. 1). Temperature was maintained constant by pumping water through the upper chamber. The eggs, which rested on the bottom of the lower chamber, were introduced through an opening in the side that was then closed by means of a lucite plug. The flow characteristics of the chamber were tested by perfusing it with a dye. The dye spread quickly and fairly homogeneously to all parts of the chamber and a flow of three to four ml./min. changed the sea water in the compartment to the extent of 90% in 1½ minutes as measured with a photo-cell. The volume of the chamber is 1.7 ml.

Radioactivity measurements. The chamber was placed on a lucite platform which in turn rested on the top of an end-window Geiger-Mueller tube; consequently the geometrical relationship between the tube and the chamber was always the same. The scaler was checked before and after each experiment against a calibrated standard. Several Geiger-Mueller end-window tubes were used during the course of the experiments. The thickness of their windows ranged from 2.3 to 3.1 mg./cm.². In the majority of experiments, corrections were made for the difference in sensitivity resulting from these differences in window thickness.

Conduct of experiments. Prior to entry into the egg chamber, the sea water was cooled by passage through coiled glass tubing in a constant temperature bath. In most experiments the temperature of the bath, and therefore in the chamber, was

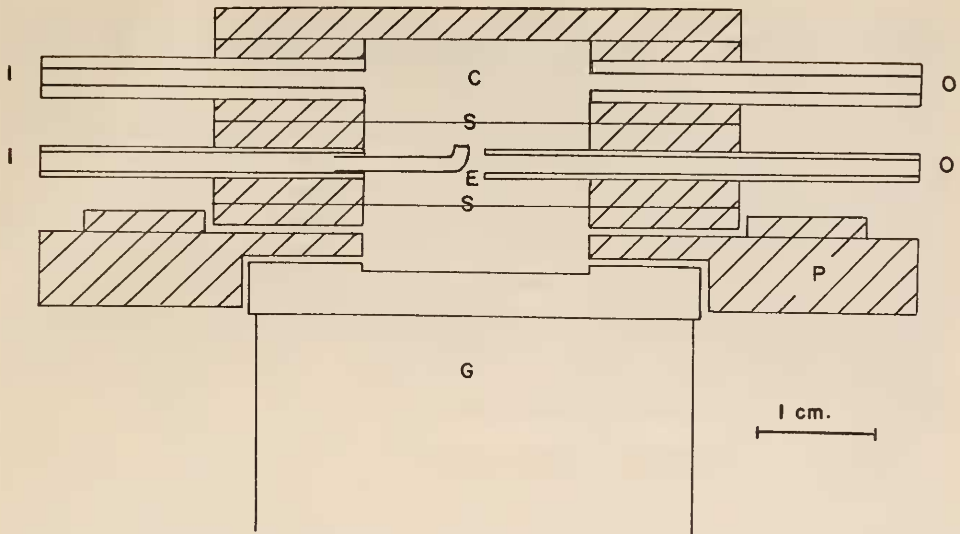


FIGURE 1. Perfusion chamber. c, cooling chamber; e, egg chamber; g, end-window Geiger-Mueller tube; i, inlet tube; o, outlet tube; p, platform; s, No. 1 coverslip.

18.0° C.; however for several experiments, the temperature was lowered to 8.0° C. Before each of these low temperature experiments, it was established with a thermocouple inserted into the chamber that the temperature of the water flowing through the egg chamber was 8.0° C.

At the beginning of each experiment, the eggs were perfused with sea water for fifteen minutes, while a background count for the experiments was obtained. Sea water containing P^{32} was then turned on and the activity in the chamber reached a new level with the unfertilized eggs. Fifty to sixty minutes after the start of the experiment, the eggs were fertilized. This was accomplished by injecting 0.1 to 0.2 cc. of a 10% sperm suspension in P^{32}-sea water into the chamber inlet tube with a hypodermic syringe. Fertilization and development of the eggs were observed with a Zeiss Opton stereoscopic microscope placed above the chamber. At the end of each experiment the eggs were removed from the chamber, washed, and allowed to develop further to check on their normality and on their recovery from the effects of any reagent that was being tested. After each experiment the chamber was rinsed alternately with concentrated HCl and NaOH and perfused with tap water and sea water in order to remove P^{32} adsorbed on the inner surfaces of the bottom compartment. Any radioactivity left in the chamber was accounted for by measuring the activity of the empty chamber just before each experiment.

In some experiments, eggs were activated parthenogenetically by the double treatment of Loeb (Just, 1939). The butyric acid and hypertonic sea water solutions were injected by means of a syringe into the egg chamber containing the unfertilized eggs. At the end of the treatment the eggs were perfused with sea water, followed by P^{32}-sea water as in the other experiments.

Materials. The eggs used were those of the sea urchin *Strongylocentrotus*

purpuratus (Stimpson). The eggs from a single animal were used in each experiment. Shedding was induced either by injection of 4.25% KCl (Tyler, 1949), or by an electric shock of 30 volts, applied intermittently for several minutes (Harvey, 1952). Eggs were shed into filtered sea water and washed until 95% to 100% fertilization was obtained. In the majority of experiments the eggs were used within one to three hours after shedding. Sperm were collected in dry Syracuse watch glasses, and suspensions were made up just before use.

A 0.5% egg suspension was prepared and 1-ml. aliquots of this suspension were counted in a Sedwick-Rafter counting chamber. Based on this count, 15,000 eggs were transferred to the experimental chamber. During transfer and distribution of the eggs to the chamber 15 to 20% of the eggs were lost, so that in each experiment approximately 12,000 to 13,000 eggs were used. This number of eggs covered the bottom of the chamber in a single layer. Control eggs were cultured at 18.0° C. during the course of the experiment for comparison with those in the chamber.

Solutions. All perfusion solutions were made up in filtered sea water. P^{32} was obtained from the Abbott Laboratories as sodium phosphate in 0.9% NaCl at pH 6.5. P^{32}-sea water solutions were made to have an activity of 0.005 μc/ml. In all but two experiments the amount of carrier phosphate added was considerably less than the 31 to 62 μg/l. normally found in sea water in this area. The additional phosphate in these two experiments had no adverse effect on the eggs. One solution of P^{32}-sea water was prepared for each experiment, and from this P^{32}-solutions containing various test substances were made. All solutions were adjusted to pH 8.

Results

Uptake of P^{32} by unfertilized, fertilized and parthenogenetically activated eggs

Experiments with unfertilized eggs confirm previous observations that there is practically no P^{32} accumulation at this time. A few minutes after the P^{32}-sea water first enters the egg chamber, the activity of the chamber reaches a level that

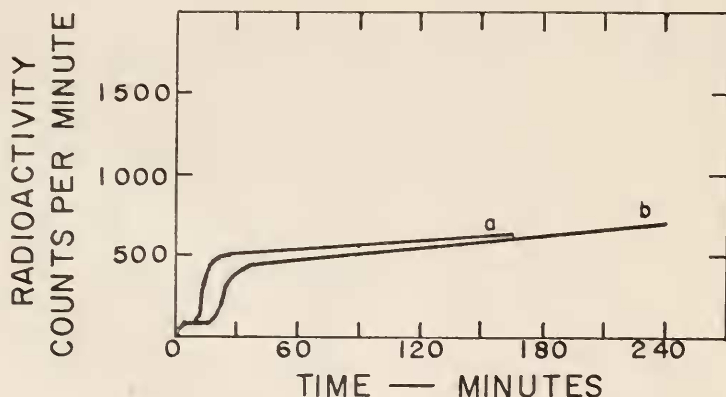


FIGURE 2. Uptake of P^{32} by unfertilized eggs of *Strongylocentrotus purpuratus* (Stimpson) and adsorption of P^{32} by the perfusion chamber. The initial low level of activity represents the background count. a, unfertilized eggs; b, empty chamber.

remains relatively constant for hours. During the course of several hours the activity may rise slightly, showing a small accumulation over the level in the sea water. The curves obtained with unfertilized eggs and with an empty chamber (Fig. 2) are very similar, from which it is concluded that the small rise is due to adsorption of P^{32} on the surfaces of the chamber and that no accumulation of P^{32} occurs in the unfertilized eggs. This conclusion is strengthened by the results of several experiments in which it was found that the rate of increase in activity in the chamber is not affected by varying the number of unfertilized eggs from 5,000 to 20,000.

In contrast to the unfertilized eggs, fertilized eggs accumulate P^{32} so that the activity in the chamber soon greatly exceeds that of the P^{32}-sea water. An experiment that typifies the course of this uptake is given in Figure 3. Two other

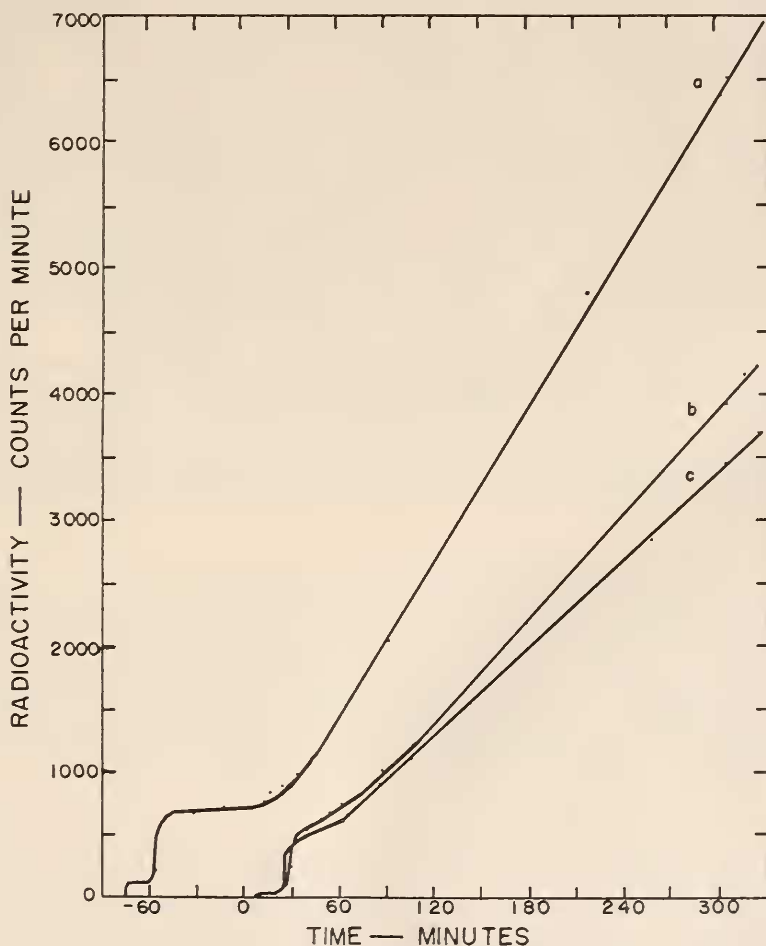


FIGURE 3. Uptake of P^{32} by sperm activated and artificially activated eggs. The initial low level of activity represents the background count. Activation was at zero time. a, sperm activated eggs; b and c, artificially activated eggs.

experiments are similar in all essential respects. This accumulation commences after a short lag period that is quite variable, ranging in 24 experiments, from 7 to 30 minutes with an average of 18. The maximum rate of uptake is not established until approximately 40 minutes after fertilization. This again is variable, ranging from 22 to 60 minutes in 14 experiments. While an explanation of these variabilities is not at hand, there seems to be no correlation between the length of the lag period and the length of time the eggs have been out of the ovary, which varied from 1 to 5.75 hours in 24 comparable experiments. It is probable that the cause resides in inherent differences in the gametes of different animals. Once the maximum rate of uptake is established, it remains constant. This constant rate of uptake was observed in experiments that continued for five hours after fertilization.

The characteristic uptake pattern of the fertilized eggs could be dependent to some degree on the penetration of the sperm, or it could be inherent solely in the potentialities of the eggs. To answer this question, eggs were activated parthenogenetically and the uptake of P^{32} followed. As is shown in Figure 3, such eggs exhibit a P^{32} accumulation comparable to that of fertilized eggs. The onset of phosphate accumulation is also preceded by a lag period. Uptake was initiated by both a single treatment with butyric acid and a double treatment of butyric acid and hypertonic sea water. The rate of P^{32} uptake in these experiments was approximately 75% of the fertilized egg rate. This difference was probably due to the activation of only 60–70% of the eggs in the chamber, as measured by membrane elevation. Moreover, none of the activated eggs cleaved normally. It seems probable that under optimum conditions, with activation approaching 100%, the uptake would approach very closely that of the fertilized eggs.

The time of onset and the magnitude of phosphate uptake appear to be potentialities of the egg, then, and are independent of the sperm. The increase in uptake does not begin until well after the visible cortical events of membrane elevation have occurred. It is of interest also that the well-known increase in respiration associated with fertilization begins earlier than the phosphate uptake and is not clearly associated with it.

Effect of low temperature on P^{32} uptake

Two experiments were carried out in which the temperature was initially 8.0° C. followed by an increase to 18.0° C. in the middle of the experiment. At 8.0° C. there was a comparatively low rate of P^{32} uptake, which was readily stimulated when the temperature was raised to 18.0° C. (Fig. 4). From these experiments a Q_{10} of 2 and 2.3 was calculated comparable to the value of 2 obtained by Villee and Villee (1952) with *Arbacia punctulata*.

Effect of 2,4-dinitrophenol on P^{32} uptake

While the establishment of the high uptake rate does not coincide with the establishment of the high respiratory rate, and the pattern of uptake during cleavage does not resemble the exponentially increasing respiratory rate, the effect of temperature still suggests that phosphate uptake may be related to energy metabolism.

2,4-dinitrophenol (DNP) is a metabolic inhibitor known to interfere especially

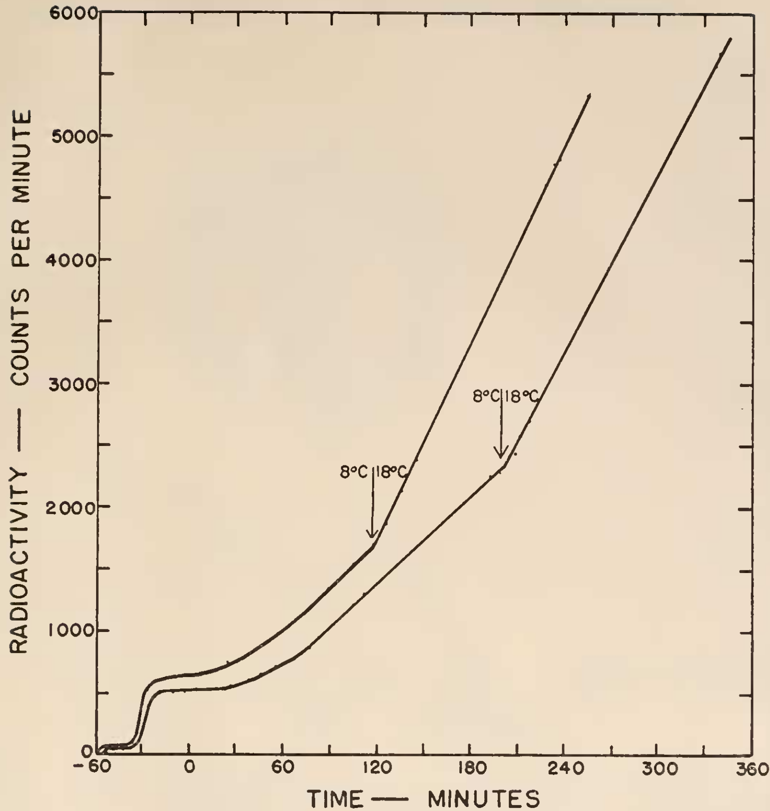


FIGURE 4. Effect of temperature on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate a change in the temperature of the perfusion solution.

with aerobic phosphorus metabolism with the result that it uncouples phosphorylations from oxidations (Loomis and Lipmann, 1948) and so in turn interferes with energy-requiring processes (Simon, 1953). Inhibition of phosphate uptake has been reported by Abelson (1947) for dinitrocresol, another substituted phenol. However, in some unpublished experiments, Whiteley had observed that when DNP was added some time after fertilization, there was no inhibition of phosphate uptake. A detailed investigation of this point has shown that the time when DNP is applied has a direct bearing on its effect on P^{32} uptake. In these experiments DNP in a concentration of 10^{-4} *M* in sea water was introduced into the perfusion chamber at various times before and after fertilization, and the effect on the rate of P^{32} uptake was measured. This concentration will reversibly inhibit cleavage. When DNP is applied before fertilization or any time within the first thirty minutes following fertilization, there is a marked inhibition of P^{32} accumulation, as may be seen in Figure 5. After thirty minutes, the effect of DNP decreases until, when it is added at sixty minutes after fertilization, it has no effect on P^{32}

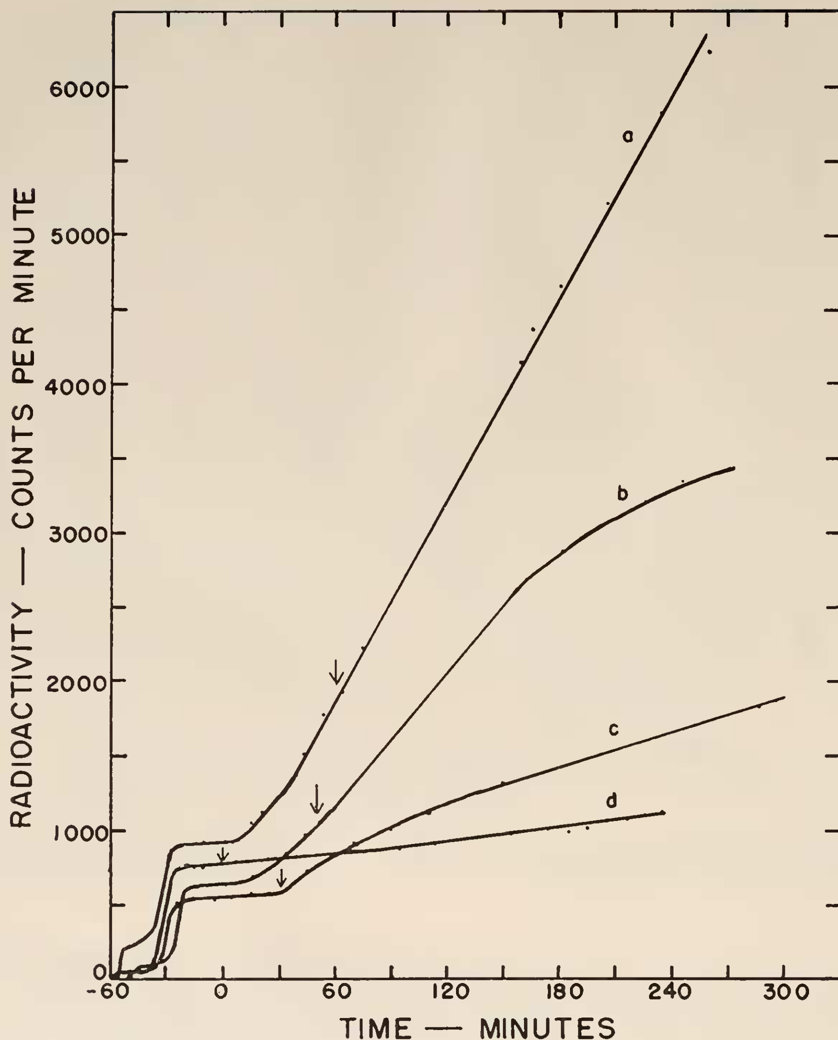
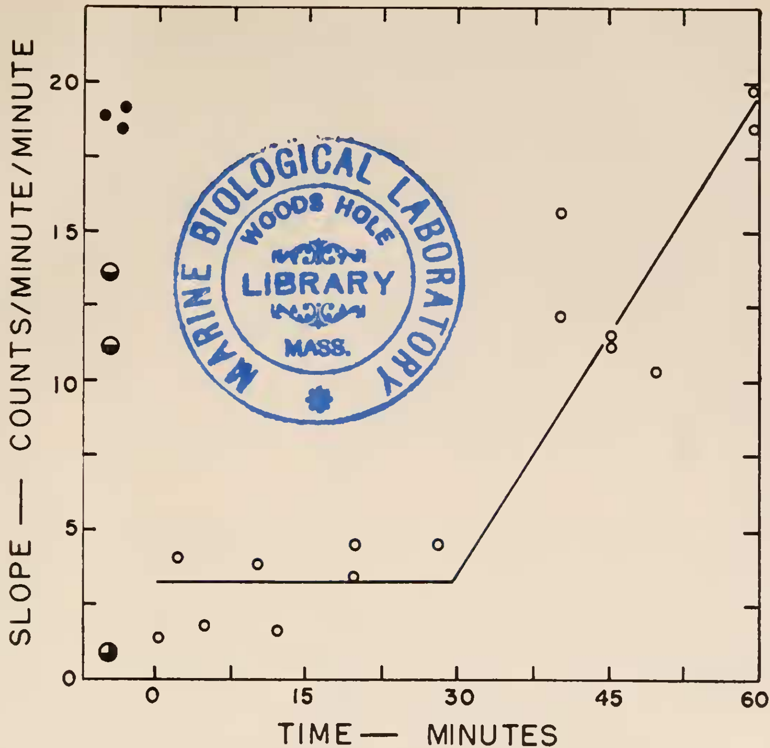


FIGURE 5. Effect of 10^{-4} *M* DNP on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate the addition of DNP to the perfusion chamber. a, DNP added at 60 min.; b, DNP added at 50 min.; c, DNP added at 30 min.; d, DNP added at insemination.

accumulation. If the rate of P^{32} uptake is plotted against the time after fertilization when DNP is applied, the time course of the inhibition can be clearly seen (Fig. 6). The maximum inhibition is associated with the first 30 minutes following fertilization. The degree of inhibition at 40 minutes is variable. This variability may be correlated with the length of the lag period and the onset of the maximum uptake rates: if the latter is not established until after the application of DNP at 40 minutes, the inhibition seems to be appreciable; if the maximum rate of uptake



MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

FIGURE 6. Effect of $10^{-4} M$ DNP on the rate of accumulation of P^{32} when the DNP is added at various times after insemination. Ordinate—average slope from insemination to the end of the experiment. Abscissa—time after insemination when the DNP was added. Open circles: fertilized eggs and DNP; solid circles: fertilized eggs; half-open circles: artificially activated eggs; quarter-open circles: unfertilized eggs.

has been established by 40 minutes, the inhibition is considerably reduced. Once P^{32} uptake has been firmly established, as at 60 minutes after fertilization, DNP has no effect on the accumulation, even in experiments continued for three hours after the application of the DNP, and despite inhibition of cleavage. Fertilized eggs are most sensitive to DNP, as far as P^{32} uptake is concerned, at a time when virtually no P^{32} uptake has been established.

Effect of adenosinetriphosphate on dinitrophenol inhibition

Kriszat and Runnström (1951), Barnett (1953) and Kriszat (1954) have reported that ATP will partially reverse the cleavage block due to DNP. In the light of these results it was thought that ATP might overcome the inhibitory effect on P^{32} uptake produced by DNP applied during the lag period. The two experiments of Table I were done to test this possibility. To conserve ATP the perfusion chamber was not used in these experiments. In each experiment four equal aliquots containing about ten thousand fertilized eggs were placed in four small flasks. At

four to six minutes after insemination the flasks received P^{32} at a final concentration of 0.01 μc/ml. and either sea water, DNP, ATP, or DNP and ATP. Final concentrations of DNP and ATP (Sigma crystalline disodium adenosine 5′-triphosphate) were 10^{-4} *M* in each instance. In Experiment 1 the final volumes were 100 ml., and in Experiment 2, 50 ml. The pH of the cultures was 7.8 to 8.0 and they were maintained with stirring at 15.5° C. Orthophosphate concentrations in the cultures varied from 2.1 to 3.8 μM. Two hundred and seventy minutes after adding DNP and ATP in the first experiment and 90 minutes in the second, the embryos were gently centrifuged and washed three times with sea water. The control eggs removed less than 12% of the available P^{32} from the culture solutions. It is clear that while DNP is markedly inhibitory when added so soon after fertilization, 10^{-4} *M* ATP does not relieve the DNP inhibition. ATP itself, in these and one other experiment, depresses uptake 15 to 28%. In both experiments cleavage was completely inhibited by the DNP, even in the presence of ATP. In the longer experiment only 5 to 10% of the embryos cleaved when subsequently placed in sea water at the end of the experiment. Recovery was 90 to 95% in the shorter experiment.

The negative results could mean that DNP damage is not repaired by direct addition of ATP, or that insufficient ATP penetrated to activate the energy-dependent reactions blocked in the presence of DNP. The permeability of developing eggs to ATP was tested directly in several experiments in which the concentration of ATP in sea water bathing eggs was measured during the first 8 or 9 hours of development. In these experiments recently inseminated eggs were suspended in sea water containing 5×10^{-5} *M* ATP (Sigma, crystalline Na_2ATP) at pH 8.0 and at 15.1° C. The egg concentrations were about 1% by volume or 10,000 eggs/ml. Aliquots of the suspensions were collected immediately and at intervals until the ninth hour of development, and were assayed for the concentrations of adenine-containing compounds (absorbance at 260 mμ), inorganic phosphate, and acid-labile phosphate (phosphate hydrolyzed in 10 minutes in 1 N HCl at 100° C.). Controls consisted of sea water with ATP, but no eggs, and sea water with eggs but no ATP.

If ATP were absorbed to or penetrated into the eggs, the absorbance of ultraviolet light and the acid-labile phosphate would diminish proportionately. If ATP were hydrolyzed to ADP or AMP by surface-located enzymes without penetration of the adenine moiety, the acid-labile phosphate would decrease, but not the ultraviolet absorbance. In different experiments the removal of adenine-containing components by the eggs varied from 0–2.34×10^{-4} μmoles/10,000 eggs/hr., and the maximum observed change in ATP that could be ascribed to a

Table I

$P^{32}O_4$ cpm/aliquot

Sample	Exp. 1 (in DNP for 270 min.)	Exp. 2 (in DNP for 90 min.)
Control eggs	6583	6642
Eggs in $10^{-4}M$ ATP	4758	5362
Eggs in $10^{-4}M$ DNP	17	42
Eggs in $10^{-4}M$ ATP and $10^{-4}M$ ATP	14	50

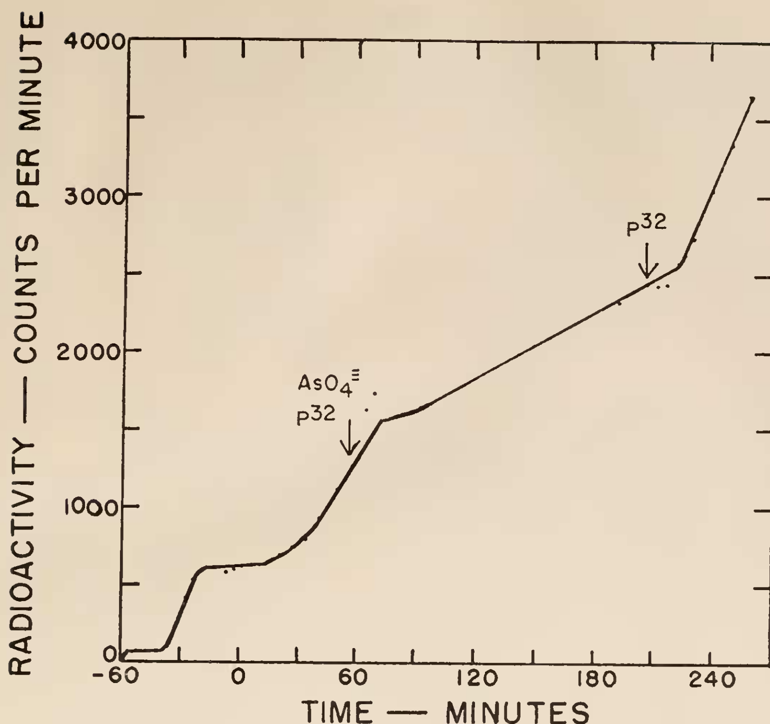


FIGURE 7. Effect of 10^{-4} *M* arsenate on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate a change in the perfusion solution.

surface-located ATPase was 7.9×10^{-4} μmoles/10,000 eggs/hr. Both these figures are at the limits of the techniques, and indicate practically no change in the ATP in the sea water around the eggs. Therefore, the failure of ATP to relieve the DNP inhibition of P^{32} uptake during the lag phase may be due to insufficient penetration of the added ATP.

Effect of arsenate on P^{32} uptake

Arsenate, because of its structural similarity, is a competitive inhibitor of phosphate in a number of enzymatic reactions.

As shown in Figure 7, a concentration of 10^{-4} *M* sodium arsenate markedly inhibits P^{32} uptake. Unlike DNP, this inhibition occurs whenever arsenate is applied from fertilization to 3½ hours after fertilization. The inhibition is completely reversible upon removal (Fig. 7, Table II). A decrease in the rate of P^{32} accumulation sets in within 1 to 5 minutes after the arsenate has first entered the chamber, and recovery from this inhibition takes place within a comparable period of time upon removal of the inhibitor. When arsenate is applied at fertilization, a limited uptake commences after a lag period that is within the range of the normal lag period, for example, 26 minutes in one experiment. Therefore,

TABLE II

Effect of 10^{-4} M arsenate on P^{32} uptake

Exp. No.	Interval after fertilization	Perfusion solution	Rate of P^{32} uptake, counts/min./min.
1	0–88 min.	AsO_4, P^{32}-sea water	6.0
	89–143 min.	P^{32}-sea water	25.0
	144–200 min.	AsO_4, P^{32}-sea water	7.0
2	0–54 min.	P^{32}-sea water	20.3
	55–204 min.	AsO_4, P^{32}-sea water	6.9
	205–260 min.	P^{32}-sea water	28.0

arsenate does not seem to increase the length of the lag period, but markedly and reversibly suppresses penetration during the accumulation phase.

It has been tentatively concluded by Yĉas (1950) that arsenate does not penetrate the eggs of the echinoid *Lytechinus pictus*. This conclusion was based on experiments that showed that arsenate had no effect on respiration or on cleavage. It was assumed that, if it had penetrated, there would have been an interference with oxidative phosphorylation, and consequently respiration and cleavage would have been affected. An experiment was set up to confirm these observations and extend them to *S. purpuratus*. Small numbers of fertilized eggs were placed in finger bowls of sea water containing sodium arsenate at various concentrations five minutes after insemination, and ninety-five minutes after insemination. The time required for these embryos to attain the early cleavages, and the normality of subsequent development were then determined (Table III). A concentration of arsenate of 10^{-4} *M* has an almost imperceptible effect on early cleavages and shows a clear retardation of development only at early blastula stages. More serious retardations set in after 8 hours in 10^{-4} *M* arsenate, leading to death in an early blastula condition at 28 hours, when controls are late blastulae. One hundred-fold stronger concentrations delay the first three cleavages by only about 20%, although development beyond 8 hours involves increasing abnormalities, with death at 22 hours. In experiments not included in Table III, 10^{-3} *M* arsenate showed retardations only a little greater than 10^{-4} *M*. Only small quantitative differences were caused by adding the arsenate at 5 minutes as compared with 95 minutes after insemination. It is doubtful that arsenate penetrates the eggs at an appreciable rate during cleavage. Therefore, the effects of arsenate on phosphate uptake are interpreted as evidence for a surface location of the penetration mechanism.

DISCUSSION

The evidence, taken as a whole, suggests the view that the rapid penetration of phosphate into sea urchin embryos is an enzymatically controlled transport. The reaction has a temperature coefficient of 2 to 2.3, which is compatible with this possibility though not, by itself, conclusive (Danielli, 1952). It is inhibited by arsenate, a competitive analogue of phosphate. Furthermore, other investigators

TABLE III

Effect of arsenate on development of embryos of S. purpuratus. Solutions were at pH 8.0 and 11.4° C., and had an orthophosphate concentration of about 2 × 10^{-6} M. Arsenate was added at 5 min. (column A) or 95 min. (column B) after insemination

Time after insemination	Controls	10^{-4} *M* AsO_4		10^{-2} *M* AsO_4	
		A	B	A	B
1 hr. 50 min.	50% 2-cell	50% 2-cell			
1 55			50% 2-cell		50% 2-cell
2 7				50% 2-cell	
2 55	50% 4-cell		50% 4-cell		
3 3		50% 4-cell			
3 7					50% 4-cell
3 25				50% 4-cell	
4 7	50% 8-cell				
4 20		50% 8-cell	50% 8-cell		50% 8-cell
4 55				50% 8-cell	
7 20	32-cell	50% 32-cell		16-cell, many abnormal	
11 35	early blastulae	very early blastulae		abnormal late cleavage	
22 35	rotating blastulae	early blastulae		dead very early blastuale	
23 20	hatching	early blastulae		—	
28 27	late blastulae	dead		—	

have found that the uptake is independent of the external phosphate concentration over a wide range (Brooks and Chambers, 1954; Chambers and White, 1954).

That the mechanism of phosphate entry is surface-located seems most probable from the results of the arsenate experiments. Arsenate must penetrate only very slowly, if at all, into the early cleavage stages. This follows from the observations of Yĉas (1950) and those reported here, that arsenate, even at the concentration of 10^{-2} *M*, shows little effect on cleavage of these eggs, and retardations occur only after some hours. The conclusion is strengthened further by Yĉas' finding that 0.05 *M* arsenate does not inhibit respiration of fertilized and cleaving eggs of *Lytechinus pictus*. In marked contrast the strong inhibition exerted by 10^{-4} *M* arsenate on phosphate uptake is evident in a very few minutes. The arsenate must have its effect at the surface by competition with phosphate for a surface-located transport mechanism, and the reaction is probably not directly linked to respiratory metabolism.

The specific reaction by which phosphate enters the embryo is not definitely elucidated by these experiments, but certain possibilities are suggested by the arsenate experiments. Arsenate is known to be a competitive analogue of phosphate, and therefore will substitute for it in enzymatic reactions. The resulting arsenate compound is usually unstable and is hydrolyzed instantly. The term

arsenolysis has been applied to the action of arsenate in splitting organic compounds (Doudoroff, Barker and Hassid, 1947).

Arsenate has been shown to inhibit the enhancement of P^{32} uptake by adenosine in mammalian red blood cells (Prankerd and Altman, 1954). In this system arsenolysis is presumed to result in a decrease in glyceraldehyde-3-phosphate. The conversion of glyceraldehyde-3-phosphate to 1-3-diphosphoglyceric acid near the surface of the cell is proposed as the mechanism of phosphate entry into the human red blood cell (Prankerd, 1956). The same mechanism could be operative in the fertilized eggs, although arsenate would also inhibit other phosphate esterifications.

The time of establishment of the mechanism is within the first 40 to 50 minutes, varying with eggs from different animals. Whether its appearance as a functional system is during the lag period of 7 to 30 minutes, or whether the subsequent period of increasing activity represents the time of establishment is not answered by these experiments. The experiments with parthenogenesis show clearly that the establishment of the mechanism is not dependent on the sperm, nor on the existence of a normal cleavage mechanism.

There remains to be considered the relation between the egg's metabolism and the transport mechanism. The presence of DNP during the first 30 minutes after fertilization prevents very markedly the later uptake of phosphate. It is assumed that DNP is exerting its characteristic uncoupling action and is consequently inhibiting the formation of high-energy phosphate by aerobic oxidations at this time. However, it appears that there is enough energy for this process at 60 minutes after fertilization despite the presence of DNP. At this time also, DNP is presumably having its effect on oxidative phosphorylation since cleavage is reversibly blocked. A correlation between oxidative phosphorylation and cleavage has been shown by Clowes, Keltch, Strittmatter and Walters (1950), whose experiments demonstrate that the concentration of a substituted phenol that will block oxidative phosphorylation in cell-free particulate systems of *Arbacia punctulata* will also inhibit cleavage in the intact egg. The conclusion drawn from this similarity in effective concentrations is that DNP inhibits cleavage by interfering with high-energy phosphate production.

Two interpretations of the effects of DNP on P^{32} uptake present themselves. According to one, the initial period may be sensitive to DNP because aerobic phosphate bond energy is needed for the synthesis of the enzymes of the transport mechanism as is the case with adaptive enzyme synthesis (Monod, 1944), or perhaps for the spatial rearrangement of the pre-formed system. The later period may be insensitive to DNP because the maintenance and operation of the mechanism requires smaller amounts of aerobic phosphate bond energy.

An alternative idea is based on a suggestion from a paper of Siekevitz and Potter (1953). It may be that the energy source for the establishment of a phosphate entry mechanism is different from that for its maintenance and operation. In experiments with rat liver mitochondria they concluded that the ATP generated within the mitochondria diffused out and mingled very slowly with that generated by glycolysis outside of the mitochondria. Consequently, there may be a separation in the functions of the ATP formed in these two locations. Synthetic reactions within the mitochondria would preferentially utilize ATP generated

locally, while an energy-requiring reaction outside of the mitochondria would be served by ATP produced by glycolysis externally. It may be that the establishment of a phosphate transport mechanism involves enzyme synthesis that is favored by ATP formed within the mitochondria. DNP could inhibit such a process by its action on the tricarboxylic acid cycle, as the enzymes for this cycle are associated with the mitochondria. Once the mechanism is established it might be maintained by high-energy phosphate resulting from glycolysis. DNP presumably does not inhibit the formation of high-energy phosphate by this means, although this has not been fully investigated (Simon, 1953). Glycolysis has been implicated in phosphate uptake by yeast (Rothstein, 1954) and by the mammalian red blood cell (Prankerd, 1956).

The negative results of the experiments testing the efficacy of externally applied ATP to overcome the early DNP inhibition are explained by the finding that ATP neither penetrates the fertilized eggs of this species, nor is adsorbed to their surface, nor is hydrolyzed at their surface in amounts detectable by the rather sensitive methods used. This failure of ATP to be efficacious is in contrast to its effect on cleavage inhibition by DNP reported by Kriszat and Runnström (1951), Barnett (1953), and Kriszat (1954). It is doubtful that the tiny amounts of energy that could have been available to the eggs, judging from the present data, would be sufficient to overcome the inhibitions as found by these investigators. If the eggs of their studies were not appreciably more permeable to ATP, one wonders if the effects could be ascribed to other substances in their ATP preparations.

According to the results of Bolst and Whiteley (1957) the rate of penetration of phosphate increases rapidly for 35 hours in the embryos of *S. purpuratus*. This is in accord with the present findings that the transport system is surface-located because, during the development to the gastrula, the number of cells, and therefore the surface area of the embryos, increases through cleavage, and it is reasonable to suppose that the newly formed surface would possess the transport mechanism.

Summary

1. The accumulation of phosphate by the eggs and embryos of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson) was analyzed by determining the action of various metabolic effectors on the uptake of P^{32} from sea water flowing at a constant rate over the eggs in a special perfusion chamber.

2. The rate of uptake of P^{32} by unfertilized eggs is nearly zero. The rate for the first 7 to 30 minutes after fertilization (lag phase) is also nearly zero, but increases rapidly during the next 20 to 30 minutes (augmentation phase), and becomes maximal 22 to 60 minutes after fertilization (accumulation phase) at a level many times that of the unfertilized eggs.

3. Artificial parthenogenesis, by either the single or double treatment, results in the same pattern and magnitude of uptake as does fertilization, even in the absence of cleavage.

4. Phosphate accumulation is markedly inhibited by 10^{-4} *M* 2,4-dinitrophenol if this agent is added during the lag phase, moderately inhibited if added during the augmentation phase, but is unaffected if added during the accumulation phase.

5. Addition of 10^{-4} *M* ATP simultaneously with dinitrophenol early in the lag phase does not alleviate the inhibition caused by the latter.

6. ATP neither penetrates into cleaving eggs nor is hydrolyzed by an ATPase at their surface.

7. 10^{-4} M arsenate markedly inhibits P^{32} uptake at all times after fertilization.

8. Arsenate does not materially retard early development of these sea urchin eggs indicating that it does not penetrate into them.

9. P^{32} uptake has a temperature coefficient of 2 to 2.3 during the accumulation phase.

10. The evidence resulting from the use of these effectors indicates that P^{32} uptake in sea urchin embryos is enzymatically controlled and that the enzymatic mechanism is located on the cell surface. The period immediately following fertilization is believed to be a time when the uptake mechanism is being established. This process appears to be dependent on phosphate bond energy, the production of which is DNP-sensitive. During the accumulation phase it is suggested that the energy requirements for the operation and maintenance of this mechanism are quantitatively much smaller, or are satisfied by phosphate bond energy the production of which is DNP-insensitive. Possible reasons for this difference in sensitivity are discussed.

LITERATURE CITED

Abelson, P. H., 1947. Permeability of eggs of *Arbacia punctulata* to radioactive phosphorus. *Biol. Bull.*, **93**: 203.

Abelson, P. H., 1948. Studies of the chemical form of P^{32} after entry into the *Arbacia* egg. *Biol. Bull.*, **95**: 262.

Barnett, R. C., 1953. Cell division inhibition of *Arbacia* and *Chaetopterus* eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds. *Biol. Bull.*, **104**: 263–274.

Bolst, A. L., and A. H. Whiteley, 1957. Studies of the metabolism of phosphorus in the development of the sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull.*, **112**: 276–287.

Brooks, S. C., 1943. Intake and loss of ions by living cells. I. Eggs and larvae of *Arbacia punctulata* and *Asterias forbesi* exposed to phosphate and sodium ions. *Biol. Bull.*, **84**: 213–225.

Brooks, S. C., and E. L. Chambers, 1948. Penetration of radioactive phosphate into the eggs of *Strongylocentrotus purpuratus*, *S. franciscanus*, and *Urechis caupo*. *Biol. Bull.*, **95**: 262–263.

Brooks, S. C., and E. L. Chambers, 1954. The penetration of radioactive phosphate into marine eggs. *Biol. Bull.*, **106**: 279–296.

Chambers, E. L., and T. J. Mende, 1953. Alterations of the inorganic phosphate and arginine phosphate content in sea urchin eggs following fertilization. *Exp. Cell Res.*, **5**: 508–519.

Chambers, E. L., and W. E. White, 1949. The accumulation of phosphate and evidence for synthesis of adenosine-triphosphate in the fertilized sea urchin egg. *Biol. Bull.*, **97**: 225–226.

Chambers, E. L., and W. E. White, 1954. The accumulation of phosphate by fertilized sea urchin eggs. *Biol. Bull.*, **106**: 297–307.

Chambers, E. L., A. Whiteley, R. Chambers and S. C. Brooks, 1948. Distribution of radioactive phosphate in the eggs of the sea urchin *Lytechinus pictus*. *Biol. Bull.*, **95**: 263.

Clowes, G. H. A., A. K. Keltch, C. F. Strittmatter and C. P. Walters, 1950. Action of nitro- and halophenols upon oxygen consumption and phosphorylation by a cell-free particulate system from *Arbacia* eggs. *J. Gen. Physiol.*, **33**: 555–561.

Danielli, J. F., 1952. Structural factors in cell permeability and secretion. *Soc. Expt. Biol. Symposia*, **6**: 1–15.

DOUDOROFF, M., H. A. BARKER AND W. Z. HASSID, 1947. Studies with bacterial sucrose phosphorylase. III. Arsenolytic decomposition of sucrose and of glucose-1-phosphate. *J. Biol. Chem.*, **170**: 147–150.

HARVEY, E. B., 1952. Electrical method of "sexing" *Arbacia* and obtaining small quantities of eggs. *Biol. Bull.*, **103**: 284.

JUST, E. E., 1939. Basic Methods for Experiments on Eggs of Marine Animals. P. Blakiston's Son & Co., Inc., Philadelphia.

KRISZAT, G., 1954. Die Wirkung von Purinen, Nucleosiden, Nucleotiden und Adenosinetriphosphat auf die Teilung und Entwicklung des Seeigeleis bei Anwendung von Dinitrophenol. *Exp. Cell Res.*, **6**: 425–439.

KRISZAT, G., AND J. RUNNSTRÖM, 1951. Some effects of adenosine triphosphate on the cytoplasmic state, division, and development of the sea urchin egg. *Trans. New York Acad. Sci.*, **13**: 162–164.

LINDBERG, O., 1949. On the turnover of adenosine triphosphate in the sea-urchin egg. *Arkiv Kemi, Mineral. Geol.*, **26B**: No. 13: 1–4.

LINDBERG, O., 1950. On surface reactions in the sea urchin egg. *Exp. Cell Res.*, **1**: 105–114.

LOOMIS, W. F., AND F. LIPMANN, 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. *J. Biol. Chem.*, **173**: 807–808.

MONOD, J., 1944. Inhibition de l'adaptation enzymatique chez B. coli en presénce de 2-4 dinitrophenol. *Annales de l'Institut Pasteur*, **70**: 381–384.

NEEDHAM, J., AND D. M. NEEDHAM, 1930. On phosphorus metabolism in embryonic life. I. Invertebrate eggs. *J. Exp. Biol.*, **7**: 317–348.

PRANKERD, T. A. J., 1956. The activity of enzymes in metabolism and transport in the red cell. *Int. Rev. Cytol.*, **5**: 279–301.

PRANKERD, T. A. J., AND K. I. ALTMAN, 1954. A study of the metabolism of phosphorus in mammalian red cells. *Biochem. J.*, **58**: 622–633.

ROTHSTEIN, A., 1954. The enzymology of the cell surface. *Protoplasmatologia*, **2**: E4, 1–86.

SIEKEVITZ, P., AND V. R. POTTER, 1953. Intramitochondrial regulation of oxidative rate. *J. Biol. Chem.*, **201**: 1–13.

SIMON, E. W., 1953. Mechanisms of dinitrophenol toxicity. *Biol. Rev.*, **28**: 453–479.

TYLER, A., 1949. A simple, non-injurious method for inducing repeated spawning of sea urchins and sand-dollars. *Coll. Net*, **19**: 19–20.

VILLEE, C. A., AND D. T. VILLEE, 1952. Studies on phosphorus metabolism in sea urchin embryos. *J. Cell. Comp. Physiol.*, **40**: 57–71.

WHITELEY, A. H., 1949. The phosphorus compounds of sea urchin eggs and the uptake of radio-phosphate upon fertilization. *Amer. Nat.*, **83**: 249–267.

YČAS, M. F., 1950. Studies on the respiratory enzymes of sea urchin eggs. Thesis. Calif. Inst. Tech., Pasadena.

ZEUTHEN, E., 1951. Segmentation, nuclear growth and cytoplasmic storage in eggs of echinoderms and amphibia. *Pubbl. Staz. Zool. Napoli*, **23** (suppl.): 47–69.